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{Exhibit 7}

John et al., "RNA-DNA Hybrids at the Cytological Level," Nature 223: 582-587 (1969)

Its essential feature is that we have shown that it is possible, contrary to usual opinion, to have a biochemically plausible theory of memory in which the information is actually stored in coded form by modifying the DNA of the cell. Our model, like the genetic switching theory^{6,7}, does give a capacity of a reasonable order ($\approx 10^{10} \lambda \log_2 p$, where λ is the number of ticked cistrons per cell involved in memory).

Finally, the matter of specificity of connexion between nerve cells is easy to incorporate into the present model, in a manner similar to the previous one^{6,7}, by supposing that the λ ticked cistrons produce λ different proteins each having specific growth-promoting ability for particular categories of neurones.

Experimental Tests

One major difficulty in the experimental search for the physical basis of memory is the small amount of chemical modification per cell, or per synapse, which may occur during a learning experiment. In the present model, for example, we cannot be sure that there need be more than a few hundred transmethylations of bases per second throughout the whole brain in order to obtain sufficient information storage per second¹⁰. Of course, this may well be a gross underestimate, but it illustrates starkly the possible magnitude of the experimental problem. Nevertheless there are certain definite lines of attack on testing the model. Methylation requires S-adenosyl methionine to provide the methyl group and therefore lack of the essential amino-acid methionine would be expected to be associated with disturbances of memory. The number of tickets per cistron might well be in the hundreds and consequently the methylated bases would tend to be clumped together in this way. Finally, one might well expect a systematic alteration with age in the number of methylated bases, probably showing a dependence on the degree of stimulation or severity of learning programmes to which the animal has been subjected.

Some observations not inconsistent with the model have already been cited. In addition, the demonstrations that

methylation appears to be the dominant determinant in the specific destruction of form DNA¹¹; that ribosomes contain an endonuclease which might qualify as the tick-tremoving enzyme¹²; and that neuronal DNA does appear to be capable of some form of limited turnover¹³, all suggest that the kind of model just presented at least warrants further exploration.

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RNA-DNA Hybrids at the Cytological Level

by

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Radioactive RNA introduced into "target" cells can be induced to form hybrids with nuclear DNA. The location of these hybrids can be detected by autoradiography.

USING RNA-DNA hybridization it is possible to detect and characterize certain specific DNA segments, for example, those specifying transfer RNA in bacteria¹, mRNA of phages², and ribosomal RNA of bacteria, insects³ and amphibia⁴. But it is not yet possible to assign the chromosomal location of a given DNA segment by means other than conventional cytogenetic and genetic analysis. Thus chromosomal mapping of sequences detectable by molecular hybridization is limited to a small number of genetically favourable organisms, or to rare viable deletion mutants⁵, or to comparisons between the sex chromosomes of some organisms⁶. Methods of genomic analysis now include the detection of families of DNA sequences by DNA renaturation kinetics⁷. This approach has shown that much of the genome of higher organisms

may consist of similar sequences repeated hundreds of thousands of times. The significance, function and chromosomal distribution of these families of DNA molecules are, however, not known. Clearly methods are required which can integrate this new molecular information within the established framework of conventional chromosome cytology.

Given complementary RNA of sufficiently high specific activity, the reiterated nature and large size of many families of DNA sequences favour both their reactivity in hybridization reactions and the detectability of the product. RNA complementary to ribosomal cistrons can readily be obtained by fractionation. Other RNAs can in theory be prepared by synthesis on an appropriate DNA template *in vitro*. Theoretically therefore many such

regions come within range of autoradiographic detection if DNA-RNA hybrids can be fabricated within nuclei and chromosomes. We have successfully developed two alternative techniques for *in situ* hybridization with HeLa cells grown in monolayer culture and *Xenopus* oocyte preparations of metamorphosing tadpoles. Radioactive RNA can be introduced into suitably prepared

uridine-5-T ($> 10,000$ m.c.p.m./ μ g) in Eagle's minimum essential medium for at least 4 days. Total (long pulse-labelled) RNA was prepared from these cells by cold and hot phenol extraction. The extracted RNA was dissolved in 4.0 ml. 0.3 M saline-EDTA*, pH 7.2, mixed with 5.1 g CsCl, and the average density of the solution adjusted to 1.70 g cm $^{-3}$. The sample was centrifuged in a fixed angle rotor for 48 h

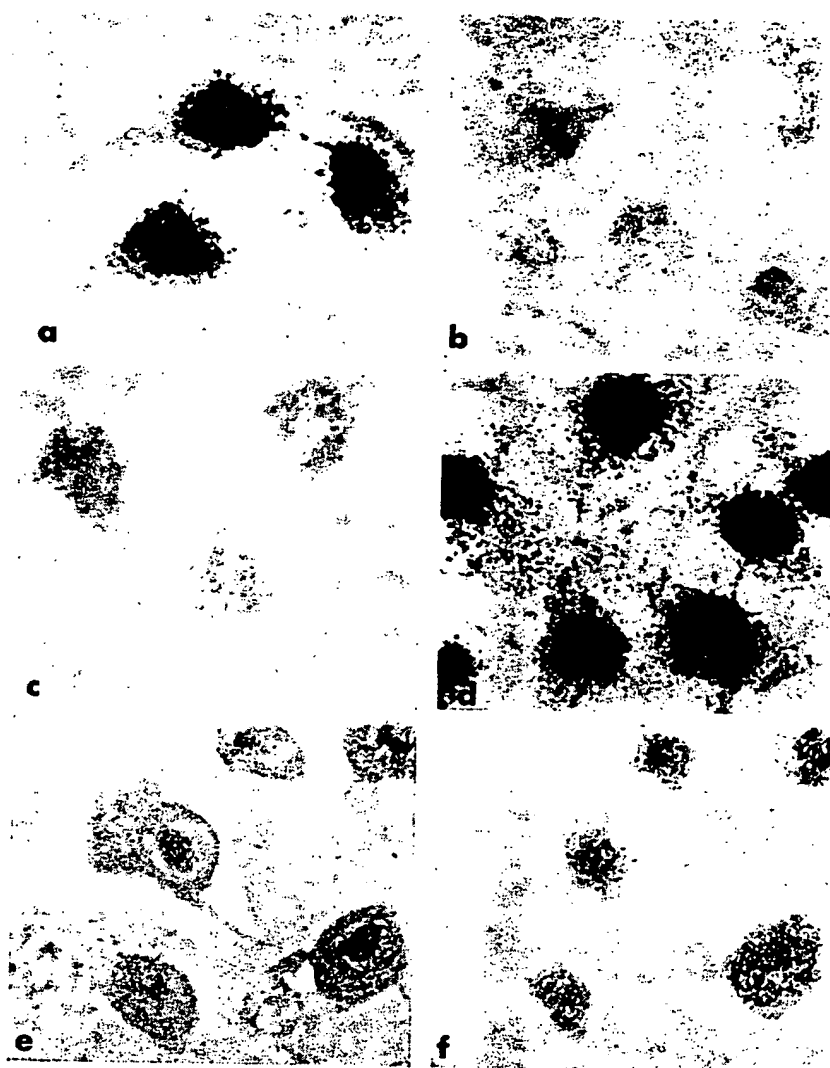


Fig. 1. Autoradiographs of intact previously unlabelled HeLa cells to show the distribution of radioactive RNA after the following treatments: *a*, reacted with HeLa total RNA at 100° C and 60° C and treated with RNase (for details see text)—note presence of RNase-resistant RNA in the nucleus and absence in cytoplasm; *b*, reacted with *Xenopus* (heterologous) total RNA at 100° C and 60° C and then treated with RNase—note marked reduction in the amount of radioactive RNA consistent with reduced homology between DNA and heterologous RNA (see Fig. 4); *c*, reacted with HeLa total RNA at 100° C and 60° C and then post-incubated in 0.1 \times SSC at 100° C for 10 min before treatment with RNase—note considerable reduction of RNase-resistant RNA consistent with dissociation of DNA-RNA hybrid to release RNA which is RNase-susceptible; *d*, similar treatment to *a* but with RNase treatment omitted—note considerable unspecific trapping of RNA in the cytoplasm; *e* and *f*, phase contrast and bright field photomicrographs of the same preparation of HeLa cells treated similarly to *a* to show intact cytoplasmic regions free of radioactivity and well defined nuclear autoradiograph. HeLa RNA 0.61 $\times 10^5$ c.p.m./ μ g. *Xenopus* RNA 0.33 $\times 10^5$ c.p.m./ μ g. Both used at 50 μ g/0.1 ml. 2 \times SSC. Exposure 6 days. Giemsa, bright field.

intact "target" cells where it can be hybridized to nuclear DNA *in situ* and detected autoradiographically. We have directly confirmed the location of amplified ribosomal DNA in the nuclei of oocytes of *Xenopus laevis* using labelled 28S RNA.

Preparation of Labelled RNA

HeLa cells and *Xenopus* kidney line cells were grown in monolayer cultures and incubated with 25 μ Ci/ml.

at 20° C at 43,000 r.p.m. to pellet the RNA and float off contaminating DNA and protein. The RNA was shown to have high molecular weight by polyacrylamide gel electrophoresis⁶. The specific activity of the RNA used was in excess of 50,000 c.p.m./ μ g. The counting efficiency of 3 H-RNA on filters was of the order of 10 per cent.

* 0.3 M saline-EDTA. 0.3 M NaCl+0.03 M sodium citrate+0.001 M EDTA+0.01 M tris HCl, pH 7.2; SSC 0.15 M NaCl, 0.015 M Na-citrate, pH 7.2.

For the preparation of *Xenopus* ribosomal RNA, monolayer cultures of *Xenopus* kidney cells which had been incubated with isotope for 5 days were chased for a 2 day period with five changes of medium. RNA was extracted and the ribosomal RNA components separated by sucrose gradient centrifugation⁹. The peak fractions at 28S and 18S were collected and the sucrose removed by filtration in 0.1 × SSC through a 'Sephadex G-25' column.

Treatment of Target Cells with Radioactive RNA after Lyophilization

HeLa cells were grown on coverslips and, while still attached to the glass, were rinsed in physiological saline, quick frozen to -70°C on dry ice and lyophilized. Radioactive RNA was introduced into the "target" HeLa cells by rehydrating previously lyophilized cells with solutions of RNA. Small (approx. 2 × 3 mm) pieces of coverslips bearing the lyophilized cells were immersed in 0.1 ml. cold 2 × SSC, pH 7.2, containing 50 μg RNA contained in a 1 ml. tube. The tube was covered and transferred to a boiling water bath for 5 min to denature the DNA, cooled rapidly in ice and then reincubated at 60°C for a further 15 min and again cooled in ice. The glass slips were then washed repeatedly in 0.3 M saline-EDTA, pH 7.2, incubated with RNase (1.0 $\mu\text{g}/\text{ml}$, 0.3 M saline-EDTA, pH 7.2, 30°C , 15 min; Worthington RNase A) to remove non-hybridized RNA¹⁰ and washed in saline-EDTA. After dehydration through alcohol series the slips were mounted on gelatine coated slides and covered with Kodak AR 10 stripping film.

For biochemical demonstrations of RNA-DNA hybrids, cells in plastic culture dishes were treated with trypsin, washed twice with physiological saline to remove trypsin, counted and pelleted. Pellets containing 5×10^6 cells were rapidly frozen to -70°C , lyophilized and then rehydrated at 0°C in 0.1 ml. RNA solution in 2 × SSC as given here. The rehydrated cells were covered with paraffin oil and incubated at 100°C and 60°C . After incubation at 60°C , 4.5 ml. cold 0.3 M saline-EDTA, pH 7.2, was then added to the chilled cells which were then homogenized in the presence of pronase (200 $\mu\text{g}/\text{ml}$, Sigma) and SLS (0.1 per cent). To release hybrids from the cells, deproteinization was allowed to proceed at 60°C for 120 min with repeated homogenization at 30 min intervals. The hybrid in the homogenate was isolated by CsCl density-gradient centrifugation in a fixed-angle rotor¹¹. 4.0 ml. of the cooled homogenate was added to 5.1 g CsCl in an MSE rotor No. 2410 centrifuge tube, mixed thoroughly and adjusted to an average density of 1.70 g cm^{-3} . After centrifugation at 43,000 r.p.m. for 48 h at 20°C , 1 g fractions were collected by piercing the tube, diluted with 4.0 ml. of saline-EDTA and the absorbancy determined at 260 mμ.

Filter-retained RNase-resistant radioactivity in the fractions was determined using procedures based on the methods of Nygaard and Hall¹² and Yankofsky and Spiegelman¹⁰. 1.0 ml. fractions were filtered through 0.45 micron HAWP 25 mm 'Millipore' filters, which were then washed twice with 0.3 M saline-EDTA before being incubated in RNase (1.0 $\mu\text{g}/\text{ml}$, 30°C , 15 min in 2 × SSC). Incubated filters were rinsed in cold saline-EDTA, dried and the radioactivity determined in a Nuclear-Chicago Unilux scintillation counter using toluene scintillation medium (2.5 l toluene, 12.5 g PPO, 0.75 g POPOP).

Treatment of Target Cells with Radioactive RNA after Acetic Acid Squashing

Ovaries from metamorphosing *Xenopus* tadpoles (stage 65) were fixed in 3 : 1 alcohol : acetic acid and tapped out in 45 per cent acetic acid on silicone treated slides. The preparation was squashed lightly under a 5 × 5 mm cover-

slip. The squash preparation was detached from the coverslip in 70 per cent alcohol, dehydrated in ethanol and dried *in vacuo*. To denature the DNA the preparation was then dipped in 0.1 × SSC at 100°C for 3 min and immediately cooled in 0.1 × SSC at 0°C , quickly dehydrated in alcohol and dried as before. The dry preparation was then placed on a drop of about 2 μl . of radioactive 28S RNA in 2 × SSC and the edges sealed with rubber solution. The sealed preparation was incubated at 70°C for 10 min to anneal the RNA to the denatured DNA, after which the coverslip was detached in 2 × SSC, washed and RNase treated (1–5 $\mu\text{g}/\text{ml}$, 30°C , 15 min, in 2 × SSC). After further washing, the coverslip was mounted on a microscope slide with the cells uppermost and stripping film applied for autoradiography.

RNA-DNA Hybridization *in situ*

After lyophilization and after being incubated as described, HeLa cell monolayers rehydrated with radioactive RNA solutions free of DNA show considerable retention of intranuclear RNA in RNase-resistant form (Fig. 1a). Note that cytoplasm is free of grains. Pellets of similarly treated cells, after lysis in detergent and suitable deproteinization, yielded RNase resistant radioactivity on CsCl gradients at buoyant densities slightly higher than that of the DNA (Fig. 2).

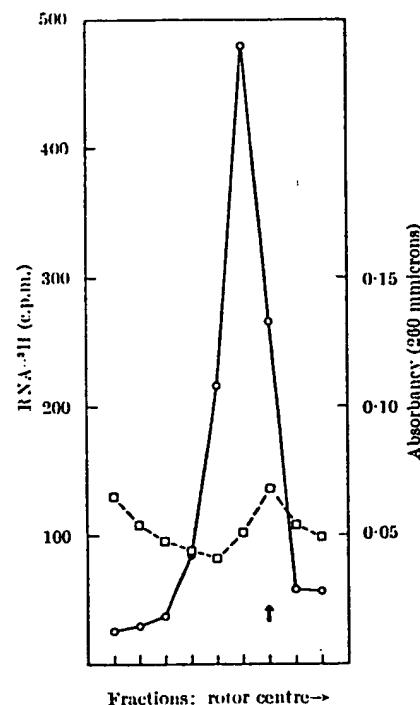


Fig. 2. CsCl density gradient analysis of the RNA-DNA hybrid recovered from intact HeLa cells. Procedures as described in text. 4×10^6 HeLa cells were reacted with 50 μg HeLa total RNA (1.3×10^4 c.p.m./ μg). □—□, Absorbance (260 mμ); ○—○, filter retained, ribonuclease-resistant radioactivity. DNA bands at a buoyant density of 1.690 (arrowed).

Denaturation of the DNA at 100°C is an absolute requirement for hybridization. Hybrids were not recovered on CsCl density gradients (Fig. 4b) when lyophilized cells were incubated at 60°C without previous denaturation of the DNA at 100°C . Similarly, oocyte cap nuclei (see here) failed to yield any significant autoradiographic grains, if the tissue had not been treated at 100°C . Heating at 60°C may, however, be omitted after heating at 100°C without affecting the recovery of hybrids (Fig.

4c). This indicates that the hybrids are formed rapidly on cooling from 100° C. Hybrids were still recovered when the temperature of deproteinization was reduced from 60° C to 0° C (Fig. 4d). In addition, when cells were reacted with radioactive RNA and the incubation mixture, after extensive dilution, treated with RNase before deproteinization, hybrid was still recovered, with a slightly increased yield in relation to the controls. This combined evidence eliminates the possibility that hybrid was formed during the deproteinization step at 60° C.

The intranuclear radioactive molecules were dissociated *in situ* and rendered RNase-sensitive by heating in 0.1 × SSC at 100° C, a treatment which is known to destroy hybrids (Fig. 1c). Likewise the DNA-associated radioactivity was rendered sensitive to RNase action as the compounds recovered from CsCl gradients were heated in SSC over a range of 60°–100° C.

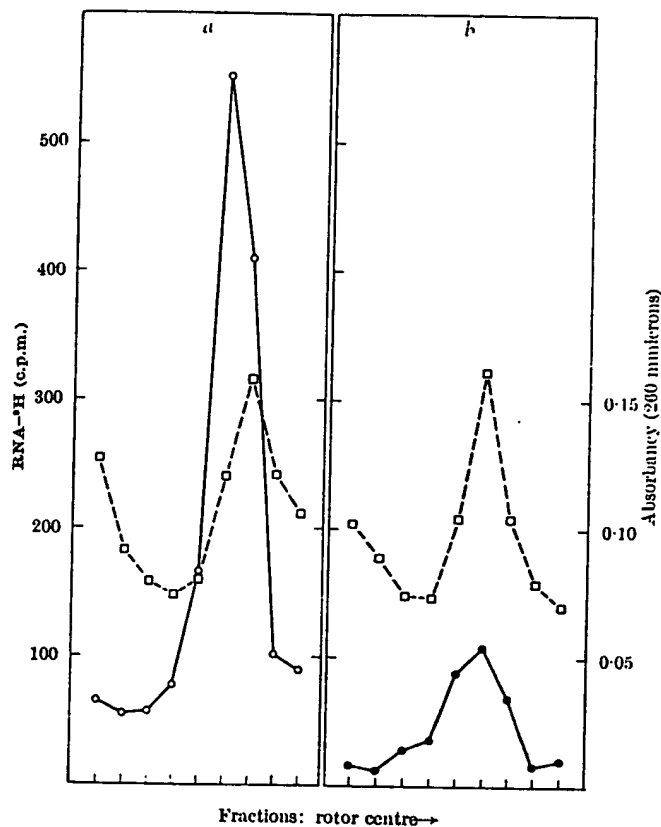


Fig. 3. CsCl density gradient analysis of the hybridization of heterologous RNA to intact HeLa cells. *a*, Control; 5×10^6 HeLa cells were reacted with 50 μ g HeLa total RNA (1.3×10^6 c.p.m./ μ g); *b*, heterologous RNA: 5×10^6 HeLa cells were reacted with 50 μ g *Xenopus* total RNA (0.33×10^6 c.p.m./ μ g). To facilitate comparison, allowance has been made for the fact that the specific radioactivity of the *Xenopus* RNA was only 25 per cent of the value for the HeLa cell RNA. In the figure, therefore, the *Xenopus* radioactivity values are increased by a factor of four. \square — \square , Absorbancy (260 mμ); HeLa 3 H-RNA, \bigcirc — \bigcirc ; and *Xenopus* 3 H-RNA, \bullet — \bullet , are values for filter-retained, ribonuclease-resistant radioactivity.

When heterologous (total, pulse-labelled) *Xenopus* RNA was introduced into HeLa cells in conditions identical to those used in homologous reactions the autoradiographic image was much reduced (Fig. 1b). Similarly the recoverable DNA-associated RNase-resistant radioactivity on CsCl gradients was drastically reduced compared with that recovered using homologous RNA (Fig. 3). Both results are expected on the basis of a reduced complementarity between heterologous nucleic acid. The dearth of silver grains over the HeLa cell nuclei when *Xenopus* RNA is used in the reaction shows that the autoradio-

graphic method is measuring the specific retention by hybridization of the input RNA.

Estimations of the extent of hybrid formation on the basis of grain yield, assuming an autoradiographic efficiency of 10 per cent¹² and a DNA content per nucleus of 7×10^{-12} g, gave a value of the order of 1–2 per cent. If allowance is made for the errors arising out of the different methods, this corresponds reasonably satisfactorily with the value of 0.4 per cent calculated for the hybrids recovered on CsCl from parallel experiments using the same sample of RNA, and supports our conclusion that RNase-resistant radioactivity demonstrated by autoradiography may be attributed to RNA–DNA hybrids formed *in situ*. The greatly decreased RNase-resistant activity recovered from CsCl gradients and in autoradiographs from heterologous reactions shows that these hybrids are highly specific (see below).

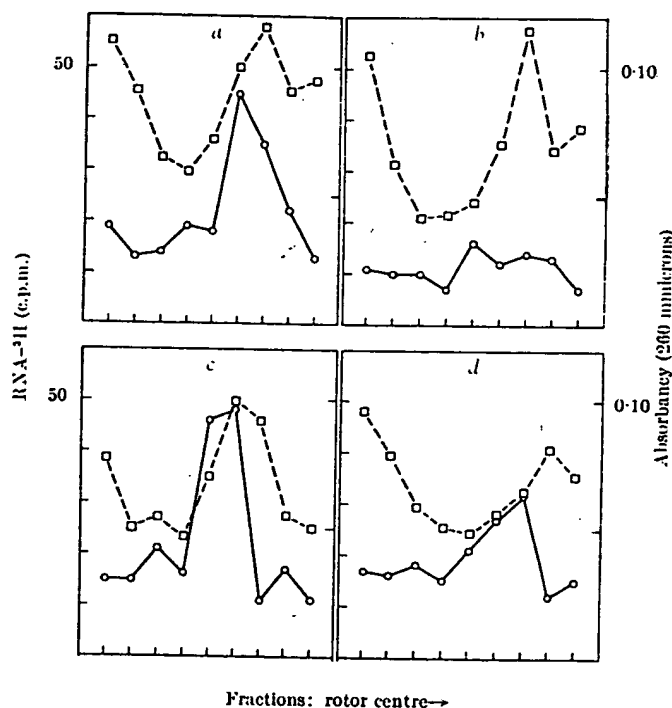


Fig. 4. Analysis by CsCl gradients of experiments in which the hybridization procedure was varied. *a*, Standard procedure (as described in text); *b*, the stage at which the cells and RNA were incubated at 100° C (DNA denaturation) was omitted; the mixture was incubated at 60° C for 15 min; *c*, the cells and RNA were incubated at 100° C but the stage at which they were incubated at 60° C was omitted; *d*, the cells and RNA were incubated at 100° C, chilled and deproteinized at 0° C. In each case 5×10^6 HeLa cells were reacted with 50 μ g HeLa total RNA (0.61×10^6 c.p.m./ μ g). \square — \square , Absorbancy (260 mμ); \bigcirc — \bigcirc , filter-retained, ribonuclease-resistant RNA.

Our studies clearly show that RNA synthesized *in vivo* anneals in hybrid form to HeLa cell nuclei in the conditions used. From work on isolated chromatin it could be suggested that nucleohistones might not have fully denatured at 100° C in 2 × SSC, because DNA bound to histones denatures some 14° C higher than does DNA which is not (or not tenaciously) bound to histones^{14,15}. If these biochemical studies on nucleohistone relate directly to the state of the DNA found *in situ*, our hybridization techniques could, in suitably chosen conditions, be extended to provide an interesting method for studying repressed and derepressed chromatin^{16–18} at a cytological level. In subsequent experiments (see here) we have used 0.1 × SSC at 100° C to denature nuclear DNA. We now use this procedure routinely and it is designed to eliminate all possible effects arising from differential melting of chromatin.

It was anticipated that renaturation of the DNA strands would be an important limiting factor in hybrid formation. We attempted to offset this by using RNA at high concentration (500 $\mu\text{g}/\text{ml}$). Despite the high concentration of RNA we suspect that DNA-DNA renaturation still effectively competes with RNA-DNA hybridization to bring the latter reaction to a rapid and incomplete end point. The fact that the hybridization occurs in a matter of seconds supports this contention.

except for the nucleolar organizer region which contains 0.012 μg of ribosomal cistronic RNA⁴.

Diploid nuclei contain two nucleolar organizers and hence 0.024 μg ribosomal cistronic DNA. The chromosomes and non-amplified nuclei therefore provide an opportunity to estimate the extent of non-specific trapping because on the basis of their content of 28S base-complementary DNA they should retain insignificant radioactivity in comparison with the caps. To make our

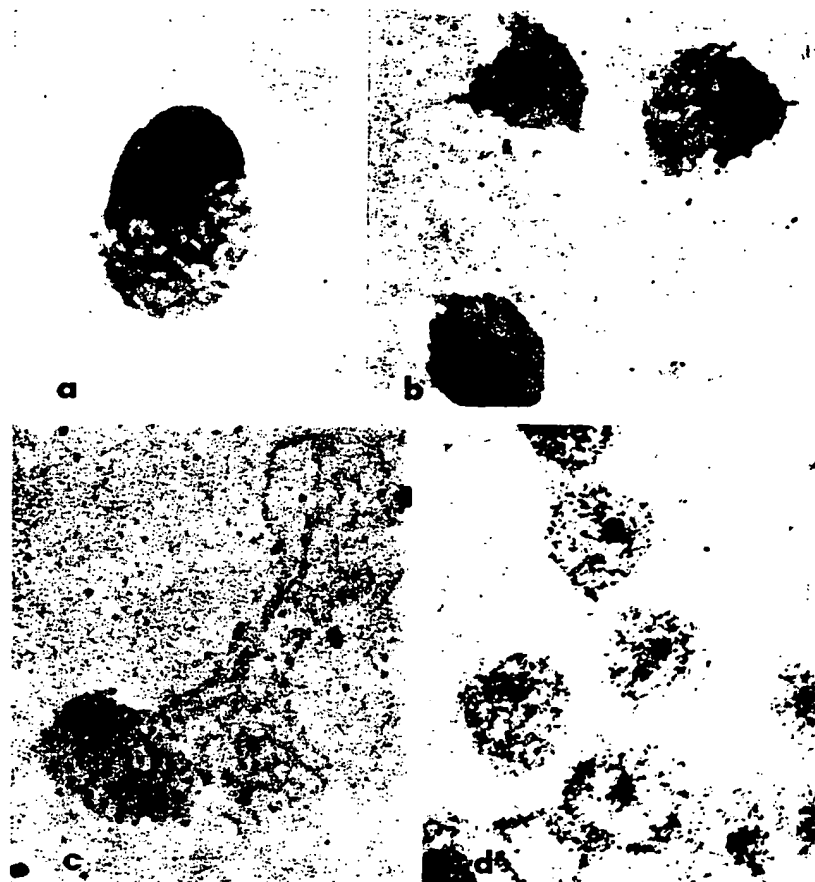


Fig. 5. *a*, Pachytene nucleus of *Xenopus* oocyte with a conspicuous cap of extrachromosomal rDNA which contains sequences homologous to 28S rRNA; phase contrast; *b*, autoradiograph of a similar preparation to *a* after hybridizing to radioactive 28S rRNA; note two nuclei with radioactive caps together with an oogonal nucleus without a cap; the latter, which does not contain amplified 28S rDNA, has no grains above background; stained with toluidine blue; *c*, squash preparation of late pachytene oocyte nucleus to show radioactive cap and non-radioactive chromosomes after hybridization with 28S rRNA; stained toluidine blue; *d*, HeLa monolayer cell preparation autoradiographed after hybridization with isolated HeLa total RNA to show preferential localization of radioactive RNA in nucleolar and peripheral chromatin. The distribution of radioactivity strikingly resembles that obtained when living cells incorporate radioactive RNA precursors. Bright field unstained. *b* and *c*, *Xenopus* 28S rRNA 0.6×10^5 c.p.m./ μg . Exposed 4 days. *d*, HeLa total RNA 0.55×10^5 c.p.m./ μg . Exposed 7 days.

There is further evidence that, despite the elacidity of the reaction, base-complementarity between RNA and DNA is the important factor in the retention of RNA within intact nuclei. The evidence comes from experiments in which purified 28S RNA was reacted with young oocytes of *Xenopus* (see here) which contain a mass of extrachromosomal DNA representing the accumulated amplified ribosomal cistrons¹⁹. In this cell the amplified DNA mass (25–30 μg) lies in the form of a cap close to the nuclear membrane (Fig. 5*a*), quite distinct from the chromosomes, which themselves contain a 4C amount of DNA, 12 μg . The cap contains a more than 1,000-fold amplification of the cistrons specific for 28S and 18S RNA and will hybridize with rRNA to approximately 18 per cent of the total mass²⁰. The chromosomes, on the other hand, contain no DNA complementary to rRNA

hybridization work comparable with previous cytological and cytochemical work, we have used the acetic acid squashing technique as an initial step for the hybridization reaction (see here). In these conditions the ovarian tissue disperses well and both caps and chromosomes are clearly distinguishable after *in situ* hybridization (Fig. 5*c*).

In our hybridization experiments we found that, whereas the caps themselves retained considerable amounts of 28S RNA (Figs. 5*b* and *c*), few or no grains were located over the chromosomes in cap nuclei or over interphase nuclei without caps. Even allowing for a DNA mass twice that of the pre-amplifying oocyte nucleus, or four times that of the somatic diploid nucleus, it is clear that the cap DNA is still vastly more efficient at hybridizing with rRNA than any other DNA found in the ovarian tissue.

The following further observations support our claim that the RNA-DNA hybrid formation is highly specific: Complementary RNA synthesized *in vitro* by bacterial RNA polymerase on rDNA⁹ anneals specifically to the nuclear cap DNA, while complementary RNA made on unfractionated *Xenopus* DNA does not. Pulse-labelled RNA from HeLa cell (1.3×10^5 c.p.m./ μ g) yields a dense autoradiograph (Fig. 1), pulse-chase labelled and purified 28S RNA from HeLa (0.9×10^5 c.p.m./ μ g) after identical exposure to the photographic emulsion does not. Furthermore, complementary RNA produced on *Drosophila* DNA hybridizes to a multitude of DNA bands in polytene salivary gland chromosomes, but *Xenopus* rRNA of similar specific activity does not (K. W. J. and F. Robertson, unpublished). For these reasons we conclude that the technique of *in situ* hybridization is a specific and sensitive method for demonstrating the cytological distribution of certain DNA sequences. While this manuscript was in preparation we heard that Dr J. Gall's group has achieved essentially similar results with different techniques^{20,21}.

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Cutaneous Vascular Changes during Heating and Cooling in the Galapagos Marine Iguana

by

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The Galapagos iguana has evolved a cooling and heating system which involves temperature sensitive cutaneous blood vessels. Local cutaneous vascular responses to temperature variations explain differences in heating and cooling rates as well as thermal stability in the face of extreme thermal loads in the environment.

THE wide range of temperatures to which the Galapagos marine iguana (*Amblyrhynchus cristatus*) is exposed during a single daily cycle has focused attention on this lizard as a valuable animal for studies of reptilian thermoregulation¹. It is clear that behavioural factors play a major part both in heat acquisition and in thermal stabilization among reptiles². That physiological mechanisms are involved is implied from observations on heating and cooling rates of *Amblyrhynchus*, which heats approximately twice as rapidly as it cools³. At any given body temperature, heart rate was slower during cooling than during heating³. The same relationships were observed for representatives of the Agamidae, Varanidae and Scincidae⁴⁻⁶; however, the ratio of cooling to heating rate for *Amblyrhynchus* appears to be the lowest (approximately 0.5). Thus far, the nature of the circulatory adjustments during heating and cooling has remained obscure. Here we present evidence, based on ¹³³Xe clearance from the subcutaneous space, that cutaneous vasomotor responses are associated with heating and cooling and that such responses are primary adjustments determining thermal transfer between *Amblyrhynchus* and the environment.

We used two marine iguanas, collected from Isla Fernandina in November 1967. Subcutaneous and rectal temperature and heart rate were monitored as animals were heated and cooled while secured to a wooden frame.

The animals were heated by an overhead infrared lamp (GE 250 W). Estimates of cutaneous blood flow were made by the ¹³³Xe clearance technique⁷. Approximately 0.05 mCi ¹³³Xe in 0.9 per cent saline (0.025 ml.) was injected subcutaneously and the rate of removal of the isotope was continuously monitored (Fig. 1).

Our initial ¹³³Xe clearance studies involved the skin area just cephalad and dorsad of the proximal portion of the hind limb. The washout curves obtained from this area were characterized by an initial slope with a faster time constant than the slower secondary component which developed about 8 to 10 min after injection. Subcutaneous injection in this site initially deposits the isotope between skeletal muscle and skin. Because of the possibility that we were observing clearances from both muscle and skin, we selected a site in the caudal half of the tail where muscle mass is minimal. Subcutaneous injection in this site gave a single exponential slope over periods of 1 h when the animal was held at a constant temperature. It should be pointed out that, once the slower constant slope component was established in the flank area, thermal stimulation induced changes in slope which were qualitatively similar to those reported for the tail site. We have not attempted to calculate the blood flow in absolute units because we have no knowledge of the partition coefficient between skin and blood⁸. We interpret our data in terms of the half-time for disappearance of the